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Monitoring sputum eosinophils on mucosal inflammation and remodelling: A pilot study

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ABSTRACT

Background: Normalization of eosinophil couts in sputum of asthmatic patients reduces eosinophilic exacerbations. However, this strategy effect on airway remodelling remains to be determined. We compared bronchial inflammation and collagen deposition after 2-years of treatment guided by either sputum eosinophils(SS) or by clinical criteria(CS).

Methods: This was a pilot study involving 20 mild asthmatics randomly assigned to CS or SS strategies. Bronchial biopsies were obtained when minimum treatment to maintain control was identified and this was continued for 2 years. They were immunostained for inflammatory cells, MUC5A and collagen.

Results: The mean dose of ICS at the start and end of the study was similar in CS and SS. The FEV_1 increased in both groups at the end of the study. In SS, mucosal lymphocyte and eosinophils counts, but not neutrophils were reduced at the end of the study. In CS, only activated eosinophils and neutrophils counts decreased. MUC5A staining decreased in SS group but not in CS. No change in collagen deposition underneath the basement membrane was observed in either strategy.

Conclusion: Treatment strategies that normalize sputum eosinophils also reduce mucosal inflammatory cells and MUC5A expression but do not change sub-epithelial collagen deposition in mild to moderate asthma.

Key Words: Airway remodelling, asthma, bronchial biopsies, sputum cell count.

Introduction

Asthma is characterized by variable flow limitation and airway hyperresponsiveness (AHR) caused by airway inflammation and structural changes (1). Bronchial biopsies have been used to evaluate the airway wall inflammatory and remodelling processes and guidelines have been published on how this assessment tool can be safely used (2,3). Although the effects of specific medications have been studied (4-6), few studies looked at the influence of various long-term treatment strategies on airway biopsies. Sont et al. showed that high doses of inhaled corticosteroids (ICS) aiming at reducing airway hyperresponsiveness were associated with a reduction of sub-epithelial fibrosis after a two-year treatment period (7,8).

Although asthma control is usually evaluated by clinical features and expiratory flows, recent observations suggest that monitoring airway inflammation by non-invasive measures such as quantitative sputum cell counts results in a reduction in asthma exacerbations (9-11). We recently conducted a study to investigate the effect of assessing asthma control and treatment needs using quantitative sputum cell counts. In this study, monitoring sputum cell counts reduced the number of eosinophilic exacerbations without increasing the total corticosteroid dose (11). As part of this investigation, we undertook a pilot study to look at the effect of treatment strategies based on sputum eosinophil count or clinical evaluation on airway wall inflammation and remodelling in bronchial biopsies over a two-year period. The expression of MUC5AC, the principal mucin produced by bronchial epithelial cells, and the thickness of collagen deposited below the epithelium were selected for the latter component of the investigation.

Methods

Subjects' selection and evaluation

Twenty subjects volunteered for this study (Table 1). At entry, asthma was confirmed by improvement in forced expiratory volume in one second (FEV_1) $\geq$ 12% after salbutamol 200μg, or by the demonstration of airway hyperresponsiveness to methacholine (provocative concentration of methacholine to cause a 20% fall in FEV_1 (PC_{20} <8 mg/ml). All subjects were either non-smokers or ex-smokers of <10 pack-years for more than six months. None had other chronic respiratory disease, a history of respiratory infection within the past two months, or other uncontrolled systemic or psychiatric diseases. The study was approved by the Laval Hospital Ethics Committee and all subjects signed written informed consent.

Study design

The subjects were those from one (Laval Hospital) of the four centres participating in a randomized controlled trial to investigate the effectiveness of using quantitative sputum cell counts (induced or spontaneous) to guide corticosteroid and antibiotic treatment. These subjects were comparable to the entire cohort in terms of demographic characteristics and sputum cell counts, but had less airflow obstruction. Patients randomized at Laval to the clinical strategy also were less hyperresponsive to methacholine compared to the entire cohort and on less daily dose of inhaled corticosteroids (Table 2). The subjects were randomized to treatment guided by symptoms and spirometry according to Canadian Consensus Guidelines (12) (Clinical Strategy, CS), or by sputum cell counts to keep eosinophils within the normal range of ≤2.0% (Sputum Strategy, SS). In both strategies, the subjects were blind to the strategy allocation and to sputum cell counts. In the CS strategy, the investigators were blind to the sputum cell counts.

In the first phase of the study, the dose of inhaled corticosteroids was adjusted to identify the minimum needed to maintain asthma control for one month. In the second phase, this minimum treatment was maintained and the subjects were seen every 3-months for 24 months from the start of the study. Adjustments to treatment were made in both phases if there was clinical deterioration and, in the SS group, if sputum eosinophils (assessed every 3 months) increased over 2%. Bronchial biopsies were obtained at the end of both phases.

Procedures

Sputum induction and processing for total and differential cell counts were performed by the methods described by Pizzichini et al (13). Bronchoscopies were performed as previously described (6). The biopsies were embedded in glycolmethacrylate monomer (Polyscience, Warrington, PA, USA) and immunostaining as previously described (14) with the following antibodies: mouse anti-human CD3, and CD4 for T lymphocytes, tryptase for mast cells, neutrophil-elastase (Dako Diagnostics, Missisauga, ON, Canada), and EG-1 and EG-2 (Kabi Pharmacia Diagnostics, Baie D'Urfay, QC, Canada), mouse anti-human Mucin 5A (Abcam, Cambridge, MA,USA) and mouse anti-human type-I collagen and mouse anti-human type-III collagen monoclonal antibodies were obtained from MediCorp Inc. (Montreal, QC, Canada). All slides were coded and sections counted blindly. Sections obtained at the end of phase 1 and 2 were processed together to maintain identical conditions. Counts were expressed as number of positive cells/mm^2 of bronchial submucosa excluding mucus glands, blood vessels and smooth muscles. The mean intra-observer variability (three repeated cell counts) was between 4-8% for all studied antibodies. Mucin 5A staining quantification was performed using Image Pro-plus software. Quantification was expressed as a percentage of staining intensity /mm^2 using color segmentation as we previously reported (15, 16). The thickness of

the collagen layer below the basement membrane was quantified using Image Pro-Plus software. These data are expressed as the mean of 3 measurements.

Statistical analysis

Clinical characteristic data were analyzed using the Student's t-tests or the Wilcoxon rank sum test for continuous variables and the Fisher's exact test for categorical variables. Values from bronchial biopsy immunostaining were log transformed to stabilize variances. The crossed-nested design was involved to analyse three experimental factors: one associated to the comparison between two clinical strategies, factor strategy (fixed) one linked to the subjects: nested random factor in strategy group and one associated to the comparison between results at baseline and two years, factor period (fixed). The factor period was analyzed as a repeated factor. A mixed model analysis was performed with interaction terms between the fixed factors. The unvaried normality assumptions were verified with the Shapiro-Wilk test and the Brown and Forsythe's variation of Levene's test statistics was used to verify the homogeneity of variances among treatments and among periods. The results were considered significant with p-values ≤ 0.05. All analyses were conducted using the statistical package SAS, version 9.2 (SAS Institute Inc, Cary, NC, U.S.A.)

Results

All 20 subjects completed the two-year evaluation. These two groups of subjects had similar clinical characteristics (Table 1). There were no significant differences in ICS doses taken by the two groups at the end of phase 1 and phase 2. There was a significant increase in FEV_1 in both groups at two years of follow-up ($p = 0.04$). Six subjects among the twenty had asthma exacerbations during phase 2 (4 in SS, two neutrophilic and two eosinophilic) and 2 in CS (neutrophilic).

We compared the percentage of sputum eosinophils in the two strategies. Eosinophils tended to be higher in the SS compared to CS (Table 1) ($p =0.06$) at baseline and were more variable ($p = 0.04$) during the duration of the study (mainly a result of the two eosinophilic exacerbations) in the SS but were similar at the end of phase 1 and after two-years in both strategies (Figure 1). Neutrophil variation was similar in both strategies (Figure 1).

We examined cell counts in the bronchial biopsies. Bronchial mucosal $CD3^+$ counts tended to be lower in CS compared to SS at the end of phase 1 (165 ± 18 compared to 263 ± 45, $p = 0.08$) (Figure 2). At the end of phase 2, these cells did not change in the CS while they decreased significantly in the SS when compared to CS ($p = 0.01$). Total ($EG1^+$) and activated eosinophil ($EG2^+$) cell counts were similar in both groups (Figure 2). However, after two-year, total eosinophils decreased from 29 ± 6 to 10 ± 4 ($p = 0.014$) in the SS but did not change in the CS (Figure 2). Activated eosinophils decreased significantly in both groups from 23 ± 11 to 8 ± 4.2 (range from 0-97 to 0-48) in the SS (Figure 2) and from 17 ± 1.3 to 4 ± 2.1 (range from 0-118 to 0-20) in the CS ($p = 0.041$. Neutrophil and mast cell counts were similar at the end of phase 1 in both strategies (Figure 3). After a two-year follow-up, neutrophils had decreased in the CS from 78 ± 16 to 58 ±18 (range: 14-151 to 1-149) (p =

0.03) (Figure 3 B) but not in the SS (figure 3A). No significant change was observed for tryptase positive cell counts (Figure 3C-D).

Mucin 5A expression was similar between CS and SS at the end of phase 1. At the end of phase 2, a significant decrease was observed in Muc5A staining in SS compared to CS ($p = 0.04$) (Figure 4A-B).

There were no significant correlations between the 2-year changes in bronchial biopsy and sputum inflammatory cells in particular between the changes in eosinophil counts in the two compartments.

Type I and Type III collagen deposition

We measured type I and III collagen deposition underneath the basement membrane in each group using techniques we previously reported (17). There were no significant changes in the thickness of the type III and I collagen layers (Figure 5) below the basement membrane between clinical and sputum groups at baseline and these parameters did not change after two-years of treatment.

DISCUSSION

In this pilot study we showed that a strategy based on a closed evaluation of asthma control according to current guidelines criteria, with or without the addition of the sputum eosinophil count to assess treatment needs, resulted in a reduction in activated eosinophils in bronchial tissue and a decrease in mucin 5A expression. However, the thickness of the collagen below the basement membrane showed no significant change.

In a recent trial, we showed that a strategy based on the assessment of sputum eosinophilia as a guide to therapy resulted in a reduction in asthma exacerbations in moderate to severe asthmatic subjects (11). There was no significant effect of this individually tailored treatment in patients with mild asthma. In the context of this large study, we evaluated a sub-group of patients to determine if there were any significant changes not only in the sputum markers, but also in the airway wall markers of inflammation and remodelling. The present study included subjects with mostly mild asthma. At the end of the study, the two groups had a similar daily dose of ICS and similar sputum eosinophil counts. This is not surprising and was comparable to what was observed in the larger study. The success of the individually titrated treatment was because we accurately established the maintenance dose of treatment at the end of phase I and we adjusted treatment during each follow-up (by increasing ICS if the eosinophils were high). This prolonged the time to the first eosinophilic exacerbation in the sputum strategy. By appropriately treating the eosinophilic exacerbation using sputum cell counts, the subsequent (and consequently, the total number of eosinophilic exacerbations) eosinophilic exacerbations were also decreased in the sputum strategy.

In the present study management of asthma according to sputum eosinophilia or clinical criteria seems to have the same impact on tissue eosinophilia. There was a significant reduction in the number of activated eosinophils infiltrating the bronchial mucosa. This

reduction may likely participate in the reduction in the release of inflammatory mediators by these cells and decrease inflammation in the bronchial mucosa. Interestingly, in the SS group T cell number in the bronchial tissue significantly decreased compared to CS strategy. However, at the end of phase one, the number of T cells in the bronchial mucosa of CS group tends to be lower than those of SS group. Thus, management of milder asthma according to sputum eosinophilia seems to have a similar efficacy as current clinical measures to control inflammation in the bronchial mucosa. In respect of asthma exacerbations, we observed similar neutrophilic exacerbations in both groups and two eosinophilic exacerbations in the SS group. However, both groups normalised their eosinophil and neutrophil counts before the second bronchoscopy.

We found no correlation between changes in bronchial biopsies and induced sputum inflammatory cells. These results are in agreement with the work of Grootendorst et al (18) who compared inflammatory cell counts in induced sputum and bronchial biopsies in asthma. They found that there was no significant correlation between eosinophil and $CD4^+$ T cell counts in sputum and bronchial biopsies. This probably reflects the fact that induced sputum eosinophilia may represent a more short-term inflammatory state of the airways, often related to environmental exposures, while bronchial wall inflammation may represent a more long-term process.

Various new strategies have been recently proposed to determine treatment needs using non invasive measures of airway inflammation such as those based on exhaled nitric oxide and induced sputum analysis, in order to improve asthma monitoring and optimize the treatment of asthma (10, 19, 20). Previously, Green et al had shown that a treatment strategy adjusting the dose of inhaled corticosteroids to reduce airway eosinophilia resulted in better asthma control when compared to the usual British Thoracic guidelines (9). There were

significantly fewer asthma exacerbations and hospital admissions with the strategy aiming at reducing airway inflammation (21). A similar effect has been observed recently when using anti-IL-5 antibody treatment (19).

While there is now good evidence that strategies targeting eosinophilic inflammation are beneficial in respect of asthma control, it is unclear whether this translates into beneficial effects on airway remodelling, a key feature of asthma. When looking at markers of remodelling, we found that mucin expression, a key feature of asthma, is reduced in SS group but not in CS group, suggesting that optimal control of eosinophilic inflammation either directly reduces or is a marker of a wider effect on the mechanisms regulating mucus secretion. These mechanisms are complex and involve cytokines produced by T cells such as IL-13 (22). Elucidation of mechanisms whereby MUC5AC staining is reduced by corticosteroids is beyond the scope of the current study but is worthy of note that in the SS group we observed a significant decrease in T cell count.

We did not observe any change in collagen 1 and 3 deposition underneath the epithelial layer over the two-year period. These features of remodelling have been shown consistently to be characteristic of asthma (23) that is resistant to corticosteroids treatment (24). A notable exception are the data from the study by Sont et al. (7) who found that using a treatment strategy targeting bronchial hyperresponsiveness as a guide to the dose of inhaled corticosteroids reduced the sub-epithelial collagen layer.

The results of this study have to be interpreted with caution as the size of the sample was small. To observe changes in sub-epithelial collagen deposition a very larger number of patients is needed. Furthermore, based on our data and previous studies (25, 26) higher doses given for longer periods would likely be needed to observe significant changes. In the study by Sont et al. the reduction of airway hyperresponsivness was achieved at the expense of

doubling of the dose of inhaled corticosteroids (7). In our study both groups received similar doses of ICS during 24 months of follow-up and were lower than those used in Sont study. In support of this explanation, Ward et al. reported significant although limited changes in airway wall fibrosis with high dose of inhaled corticosteroids for 52 weeks while the other parameters improved more rapidly (8).

The benefits of reducing airway remodelling may be to reduce the long-term decline pulmonary function or reduce the severity of the disease although this is still controversial. Monitoring airway eosinophilia may at least reduce exacerbations and improve asthma control as shown by Green et al and others, and while the beneficial effects of acting on remodelling have to be further studied before recommending a strategy aimed at reducing this process. It is possible that the strategy used changed other components of extracellular matrix that we did not measure, such as the content of proteoglycans. Recently, we showed that mast cells stimulate bronchial fibroblasts obtained from asthmatic subjects but not from normal subjects to produce procollagen through IL-4 production (27). In the present study, we found no significant changes in mast cell count over the two-year follow-up. Ward et al previously reported that variation in sub-epithelial fibrosis was related to variations in the percentage of mast cells in the bronchoalveolar lavage and suggested that mast cells might be associated with the development of airway remodelling (28). However, the ICS doses used in our study, and determined by asthma control level instead of an arbitrary dosing, may not be sufficient to observe a change in these parameters.

In conclusion, this study reports important data on the comparative influence of clinical and induced sputum strategies to assess medication needs in mild to moderate asthma. It shows a significant reduction in some markers of inflammation and mucin production but no change in sub-epithelial collagen deposition. It would be of interest to further study the

influence of these strategies in patients with higher baseline eosinophils levels and more severe disease or earlier in the course of the disease. Finally, it might be that the best strategy will be one in which eosinophilia is used as a target to reduce the exacerbation rate while hyperresponsiveness is used as a target aimed at reversing airways remodelling.

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Figure legends

Figure 1: Variation in sputum eosinophils (●) and neutrophils (●) during the twenty-four month follow up in SS and CS. (arrows indicate bronchoscopy)

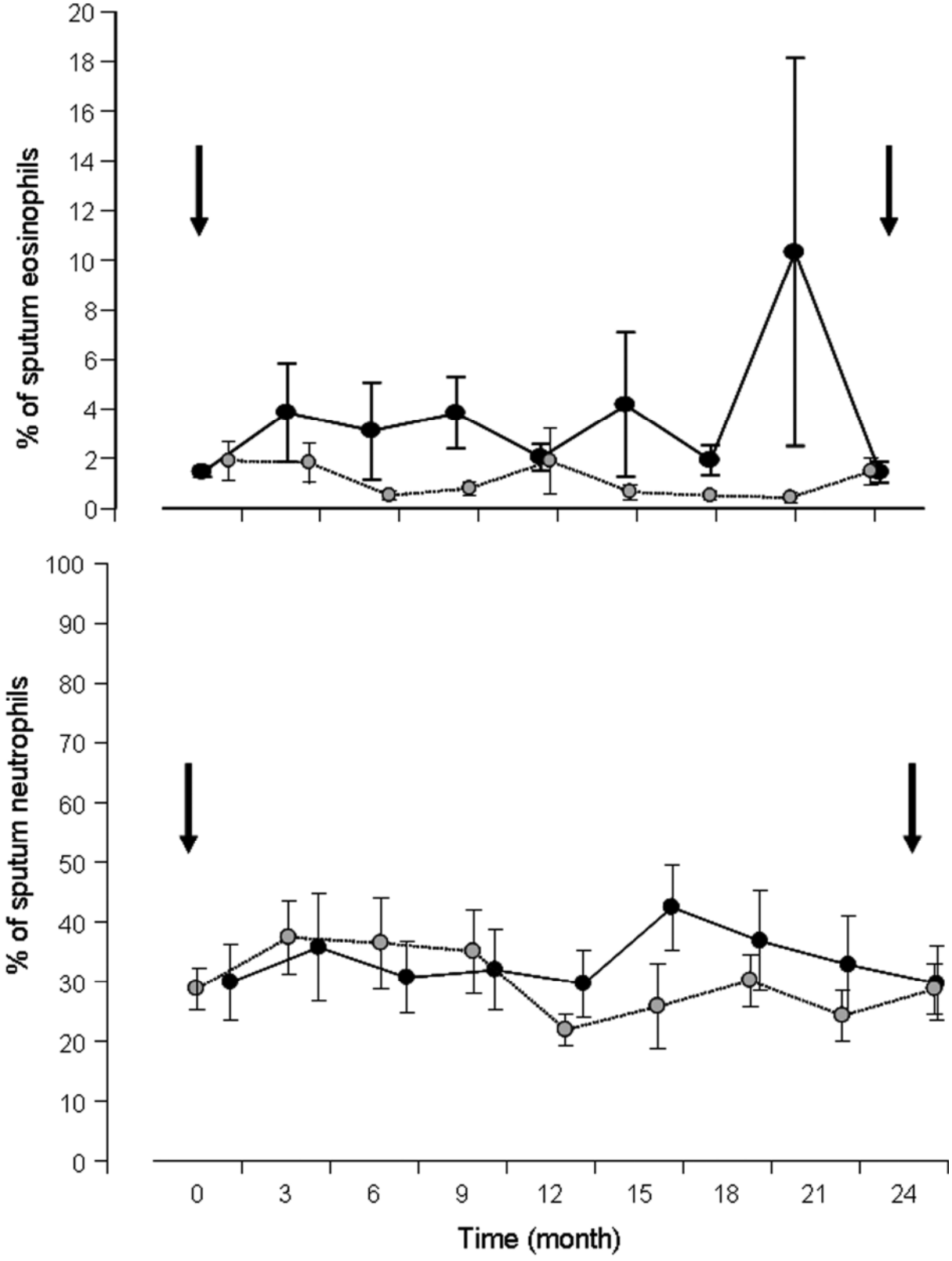
% of sputum eosinophils
20
18
16
14
12
10
8
6
4
2
0
% of sputum neutrophils
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
Time (month)

Figure 2: $CD3^+$ cells, total eosinophils (EG1) and activated eosinophils (EG2) measured by immunostaining of bronchial biopsies in SS (A, C, E) and CS (B, D, F) Results are presented as mean values of positive cell counts/ mm^2 of connective tissue.

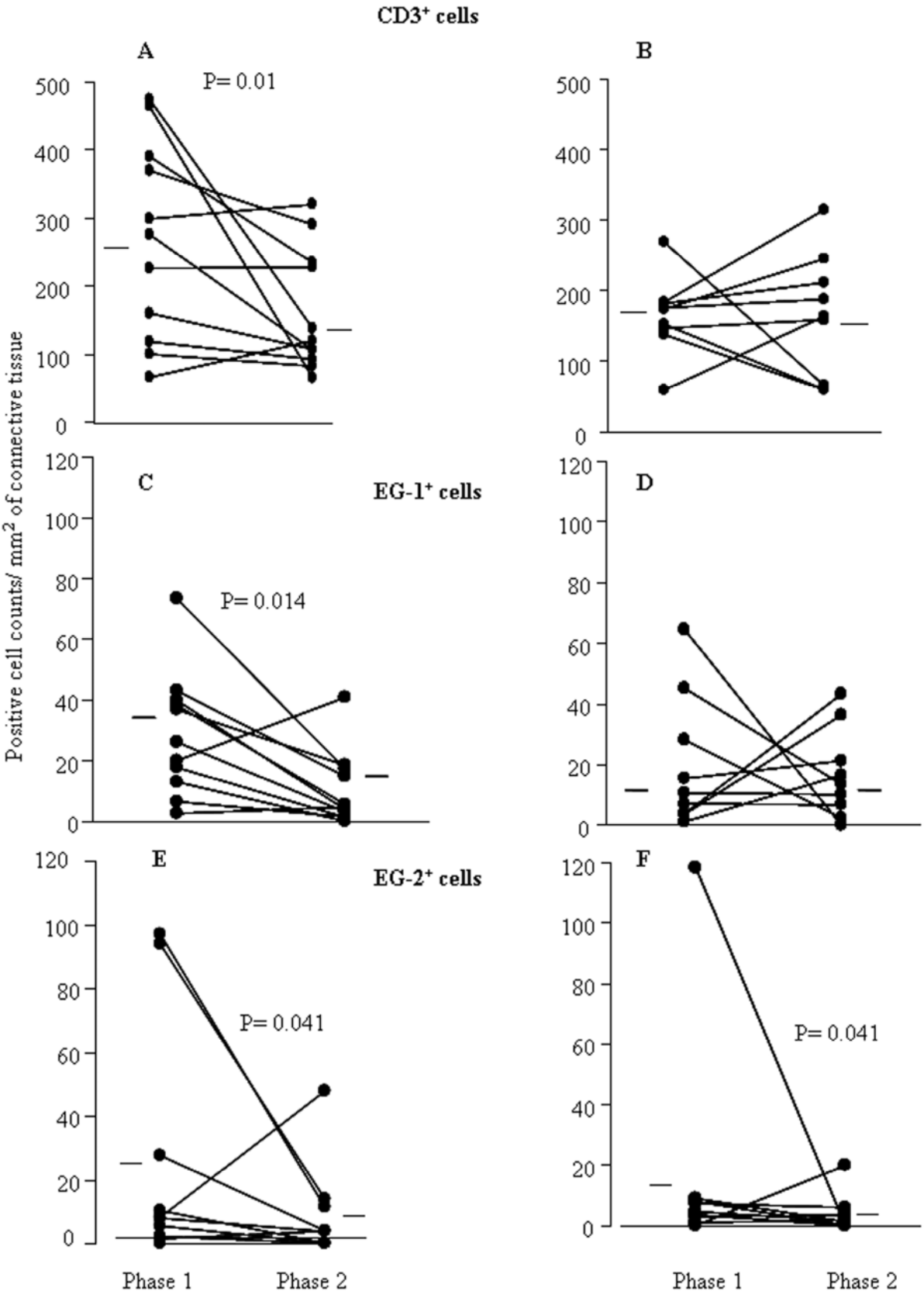
CD3+ cells
A
P= 0.01
B
C
EG-1+ cells
P= 0.014
D
E
EG-2+ cells
P= 0.041
F
P= 0.041
Positive cell counts/ mm2 of connective tissue
Phase 1
Phase 2
Phase 1
Phase 2

Figure 3: Neutrophil (elastase positive cells) and mast cell (tryptase positive cells) counts measured by immunostaining of bronchial biopsies in SS (A and C) and CS (B and D). Results are presented as mean values of positive cell counts/ mm^2 of connective tissue.

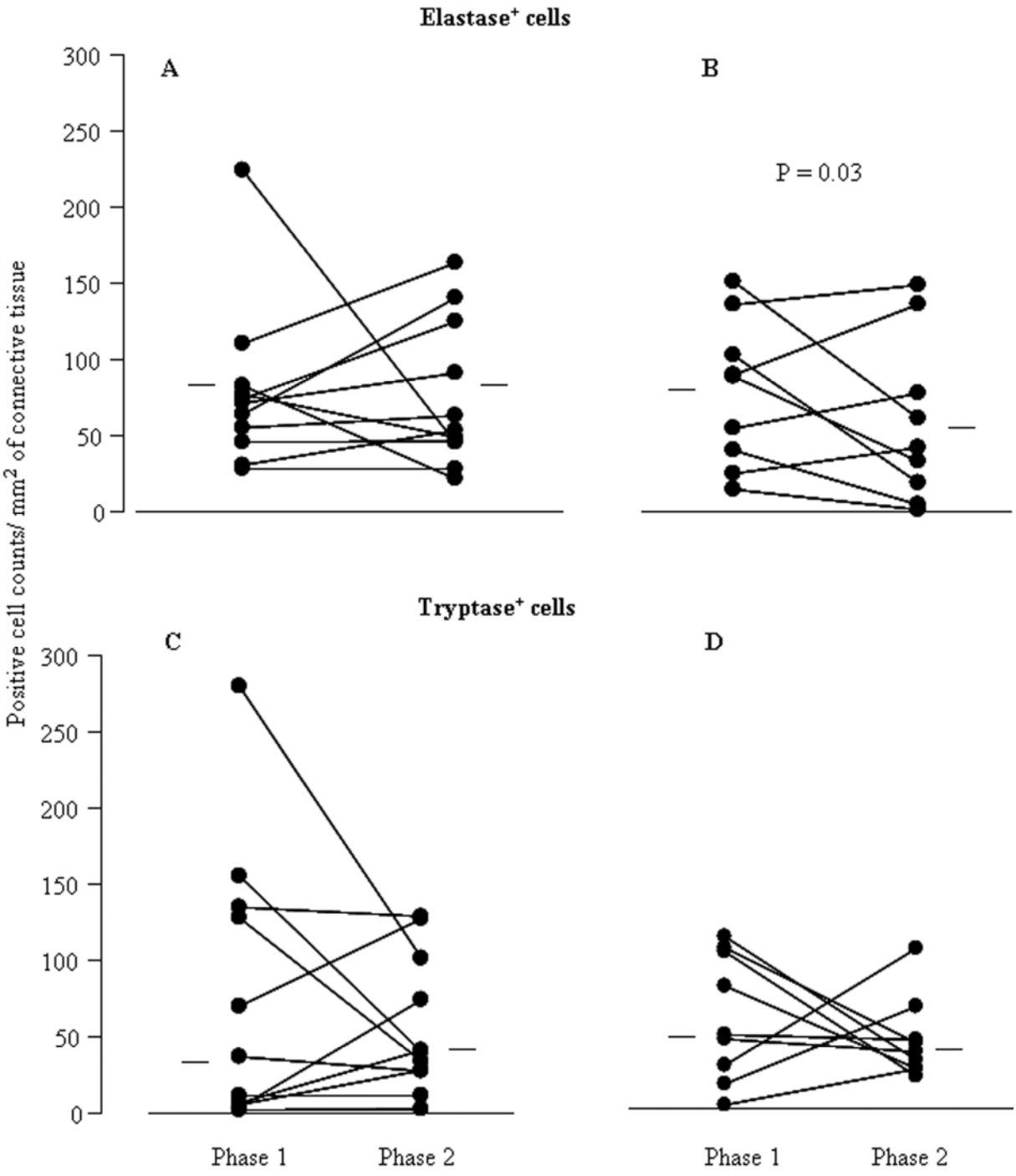

Figure 4: Muc5A staining in SS (A) and CS (B). Results are presented as percentage of staining intensity /mm^2

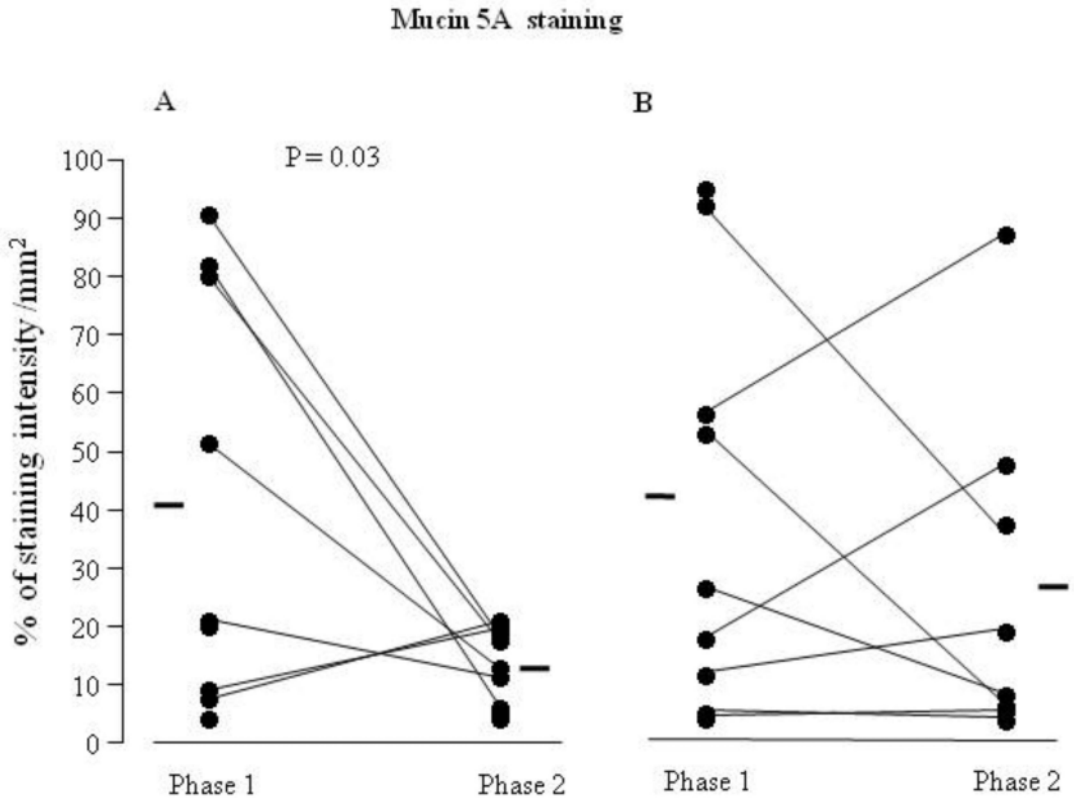

Figure 5: Sub-epithelial thickness of type I and III collagens in SS (A and C) and CS (B and D).

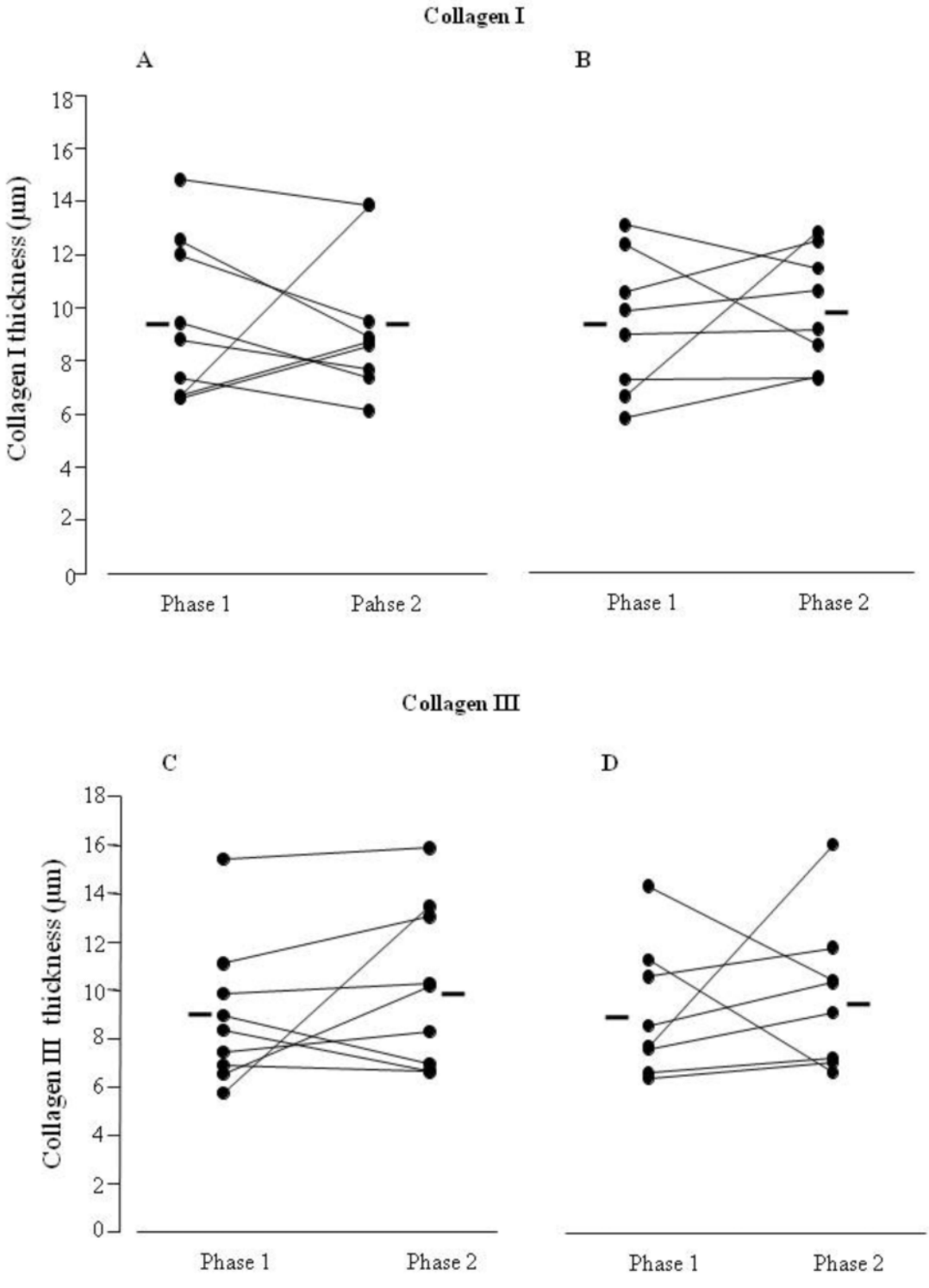
Collagen I
A
B
Collagen I thickness (μm)
18
16
14
12
10
8
6
4
2
0
Phase 1
Pahse 2
Phase 1
Phase 2
Collagen III
C
D
Collagen III thickness (μm)
18
16
14
12
10
8
6
4
2
0
Phase 1
Phase 2
Phase 1
Phase 2

Table 1: Subjects' clinical characteristics at baseline and the end of phase 1 and 2

	Sputum strategy(SS)			**Clinical strategy (CS)**		
	Baseline	Phase 1	Phase 2	Baseline	Phase 1	Phase 2
Number	11			9		
Gender (F/M)	7/4			4/5		
Age (y)	38 ± 4			41 ± 6		
Asthma duration (y)	19 ± 5			21 ± 5		
Atopy (%)*	100			90		
ICS (µg) †	445.4±80.4	523 ± 84.6	505 ± 89	420.±117	484 ± 215	421 ± 91
PC_{20} (mg/ml)	1.85±0.90	1.51 ±0.7	2.10 ± 0.48	3.33±1.07	3.93 ± 1.14	2.64 ±0.6
FEV_1 (% pred)	83.9 ±4.6	80.6 ±5.5	87.5 ± 5.9[§]	82.4 ±10.1	85.3 ± 4	89 ± 4.8[§]
Eosinophils %	4.6 ±1.6[#]	1.7 ±0.97	2 ± 1.5	1.3±0.4	1.5 ± 1.8	1.5 ± 1.6
Neutrophils %	28.2±6.8	33 ± 21	32 ± 20	21.2±4.6	26.1 ±11.2	27 ± 16

All values expressed as mean ± sem.
*At least one positive skin prick test (≥ 3-mm wheal at 10 min).
† Mean daily use of beclomethasone dipropionate or equivalent.
[§] p = 0.04 compared to baseline
[#]p =0.06 compared to CS at baseline

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